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Differentiation Between Acute Primary and Recurrent Human Cytomegalovirus Infection in Pregnancy, Using a Microneutralization Assay

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Acute primary human cytomegalovirus (HCMV) infection in pregnancy, the major cause of congenital symptomatic infection, is often difficult to differentiate from recurrent infection, which presents a considerably smaller risk to the fetus. Therefore, the diagnosis of primary infection in pregnancy is very important, especially if seroconversion is not documented and follow-up sera with declining IgM-titers are not available.

To investigate the value of the neutralizing antibody response against HCMV in differentiating acute primary from recurrent and past infection, well-characterized sera from pregnant women were examined. Employing a microneutralization assay, it was found that neutralizing antibodies first appeared approximately 15 weeks after acute infection. However, serum samples of pregnant women with recurrent or past infection consistently displayed neutralizing activity. In conclusion, the neutralization assay can be used as a reliable method for discriminating acute primary from previous or recurrent infection in a single serum sample. ***J. Med. Virol. 56: 351–358, 1998.***

KEY WORDS: HCMV; neutralizing antibodies; primary infection in pregnancy

INTRODUCTION

Human cytomegalovirus (HCMV) is the most common cause of congenital infection, affecting about 1% (0.2– >3%) of all live births in the United States [Alford et al., 1990; Demmler, 1991]. Although 90% of infants are asymptomatic at birth, about 10% develop cytomegalic inclusion disease, with a 20–30% mortality rate [Grose et al., 1989]. However, up to age 2 years, 10–15% of the children asymptomatic at birth develop neurologic sequelae [Porath et al., 1990], with hearing loss observed most commonly [Williamson et al., 1992; Fowler et al., 1997].

Because fetal damage is associated most frequently with asymptomatic maternal primary infection as opposed to recurrent infection [Nelson and Demmler, 1997], serological diagnosis of HCMV infection differentiating between a primary or recurrent infection in pregnancy would be of major importance. In general, the serological diagnosis of primary HCMV infection is based on seroconversion, a significant rise in IgG antibody titer, the detection of significant concentrations of specific IgM, or a combination of these criteria. However, appropriately timed sera are not available from most women, and the presence of specific IgM alone cannot be used to identify primary HCMV infection, because specific IgM responses may persist for long periods and may also occur after HCMV reactivation or reinfection. Hence, there is a need for supplementary assays which are able to distinguish between primary and recurrent or previous HCMV infection. Different types of such supplementary assays have been employed: an enzyme immunoassay (ELISA) using two immunogenic recombinant proteins, p52 and p150 (first described by Landini et al. [1989]), for the detection of IgG antibodies has been used in our laboratory since 1993, and proved to be helpful in identifying primary infection within the first 8 weeks (Daiminger et al., 1998). Another approach is the detection of low-avidity IgG antibodies up to 12–18 weeks after primary infection [Grangeot-Keros et al., 1997; Lazzarotto et al., 1997]. However, both methods require paired sera, and furthermore, Lazzarotto et al. [1997] showed that, using the avidity test, some primary infections may escape detection.

Recent publications [Kropff et al., 1993; Urban et al., 1996; Schoppel et al., 1997] described the application of recombinant ELISA to study the development of neutralizing antibodies to gB and gH. However, commercial kits are not available at present. Therefore, the neutralizing response was studied by a microneutralization assay using the HCMV reference strain AD169. The capacity of the neutralization assay to differenti-

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ate primary from recurrent infection in a single serum was investigated as well as the time of infection, using follow-up sera.

MATERIALS AND METHODS

Serum Samples

A total of 370 sera obtained from 198 pregnant women at various stages of pregnancy was collected from May–December 1997.

In group I, follow-up sera (n = 54) from 12 women with documented HCMV-seroconversion were investigated.

Group II comprised 235 sera from 162 previously infected women. The majority were negative for HCMV-IgM antibodies, and in some, persisting IgM at a low titer were observed over a long time period.

Group III consisted of 81 samples from 24 pregnant women which could not be differentiated readily using standard serological methods because of a positive IgM-response in two IgM-tests or a highly positive IgM-response in at least one of two tests.

All samples were collected and stored at –20°C prior to testing.

Serological Analysis

For HCMV IgG detection, the Enzygnost Anti-HCMV IgG Indirect EIA (Behringwerke, Marburg, Germany) was used, and the test was carried out with the automatic ELISA processor Behring BEP III (Behringwerke). The IgG values are expressed as PEI units/l (Paul-Ehrlich-Institut, Langen, Germany). Sera with ⩽180 PEI units/l were considered negative. Sera with an OD >2.0 (approximately 10,000 PEI U/l) were retested in dilution of 1:10.

HCMV-specific IgM antibodies were detected by the Stuttgart "in-house" modification of the enzyme-labeled antigen IgM ELA (HCMV IgM-ELA, Medac, Hamburg, Germany) and the μ-capture enzyme immunoassay ETI-CYTOK-M reverse (Sorin, Saluggia, Italy). The HCMV IgM ELA is a two-step enzyme immunoassay based on the μ-capture principle. Serum specimen and calibrators are incubated with a monoclonal anti-human IgM antibody (DAKO, Hamburg, Germany) on the solid phase of microtiterplates (Nunc, Roskilde, Denmark). Bound human antibodies are labeled during the second incubation with HCMV antigen coupled to horseradish peroxidase. For the test, the automatic ELISA processor Behring BEP III (Behringwerke) and the washing buffers from the Enzygnost Anti HCMV IgM kits (Behringwerke) were used. IgM titers of ⩽1:32 are considered negative, titers of 1:64–1:256 low positive, titers of 1:512–1:2,048 positive, and titers of ⩾1:4,096 high positive.

Microneutralization Assay

A modification of the microneutralization assay as described by Gonczol et al. [1986] was carried out without complement enhancement. Human embryonic lung fibroblasts (passages 14–17) were seeded in 96-well microtiter plates (2×10^4 cells/well) 1 day before testing. All serum-samples were heat-inactivated (56°C/30 min). Fifty microliters of serum (dilutions 1:4–1:512 in minimum essential medium (MEM) without fetal calf serum (FCS)) were incubated in duplicate with 50 μl of a constant titer of AD169 virus stock (50 immediate-early antigen-producing units (IEU) per well). After 90 min of incubation at 37°C, the virus/serum mixture was added to the monolayer and centrifuged at 1,200 × *g* for 30 min. The shell vial culture was allowed to incubate overnight (16–18 hr) in a moist chamber at 37°C with 5% CO_2 in air. Subsequently the cells were fixed for 10 min with ice-cold acetone/methanol (40:60), then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 min and afterwards incubated for 30 min with a mixture of murine monoclonal antibodies against 72-kD IE-protein and p52, a 43-kD early-protein diluted 1:100 in PBS (DAKO, Glostrup, Denmark). After washing (3 times with PBS), the plates were incubated with a horseradish peroxidase-labeled antibody at a dilution of 1:1,000 in PBS (anti-mouse-HRP, DAKO, Glostrup, Denmark). The washing step was repeated, and HCMV-positive nuclei were stained using the substrate 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, MO) and counted microscopically. The serum dilution producing 50% inhibition of virus infectivity in comparison to an untreated control was reported as the neutralizing titer.

Virology

Virus detection in urine of newborns was attempted within the first 2 weeks of life by inoculation of human embryonic fibroblasts, by the rapid shell vial assay, and sometimes also by PCR [Daiminger et al., 1994]. The rapid shell vial assay was carried out as follows: 1 ml of urine was sterile-filtered using an Amicon filter (Centricon 100, Amicon, Inc., Beverely, MA), and the nonfilterable remainder, carrying cells and virus, was resuspended in 500 μl of culture medium. Subsequently, 200 μl of this suspension were centrifuged at 1,200*g* for 30 min onto 96-well microtiter plates seeded with human embryonic fibroblasts. Detection of infected cells was carried out after 18 hr, as described above for the microneutralization assay.

Homogenized abortion material was inoculated on human embryonic fibroblasts and additionally investigated by PCR. The cell cultures were examined twice a week for cytopathic effects and discarded after 4 weeks.

RESULTS

HCMV-neutralizing antibodies were determined using a microneutralization assay. In the first of the follow-up sera of 12 women who seroconverted (group 1), no neutralizing capacity against the reference HCMV strain AD169 was detected. As shown in Figure 1A, the first neutralizing titer appeared after an average of 15 weeks (range, 14–17 weeks). The neutralizing titers tended to be low initially (1:4–1:32) and were seen to increase in follow-up sera from 1:64–1:128. The time point of seroconversion for 3 women was known precisely (see Fig. 2). In 2 cases an IgG-negative, but IgM-

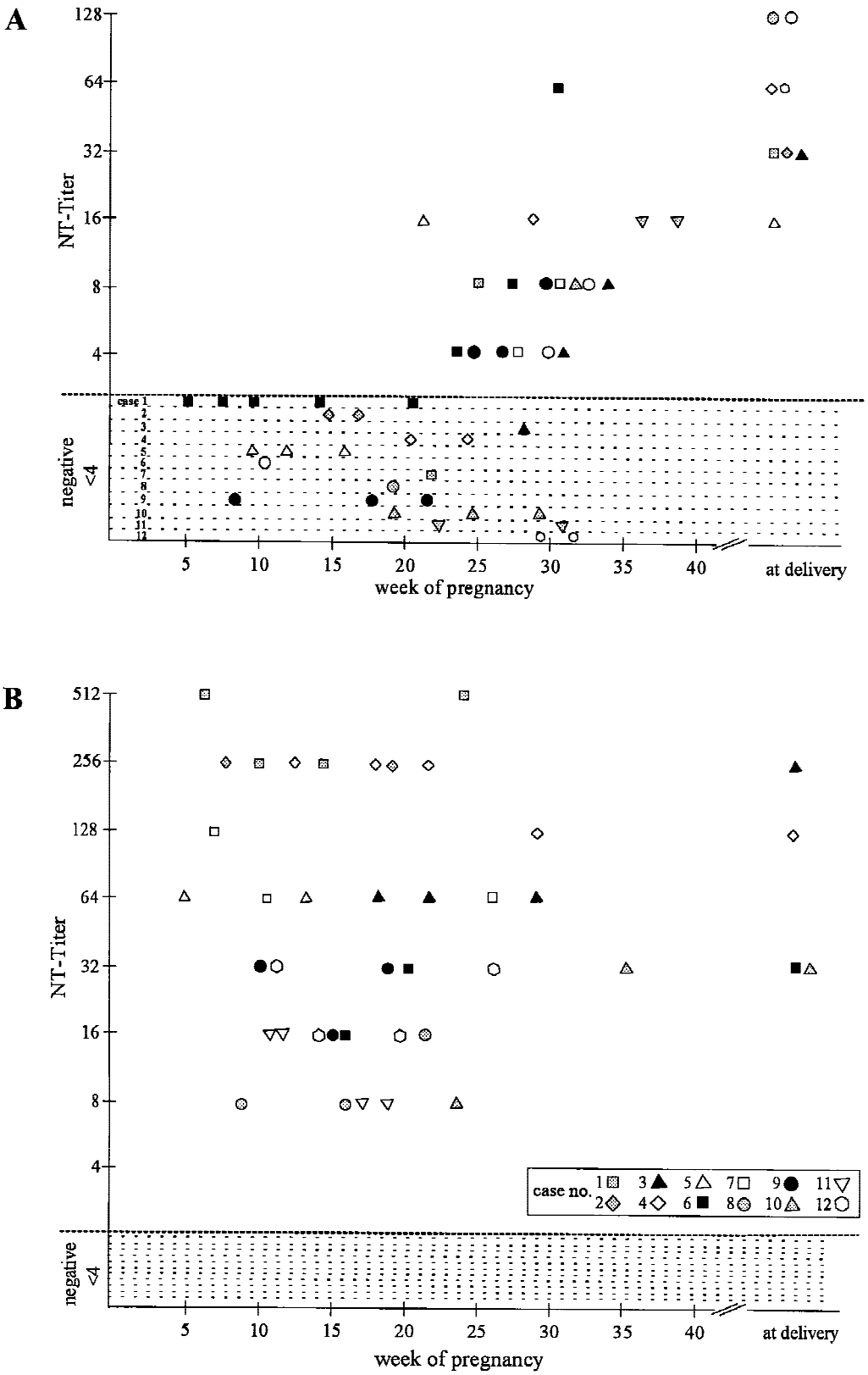

Fig. 1. Comparison of neutralization antibody titers of follow-up sera from 12 pregnant women with HCMV seroconversion (group I) **(A),** and of follow-up sera from 12 representative pregnant women with previous HCMV infection (group II) **(B).** Sera without NT-antibodies but with a positive IgG- and/or IgM-antibody response are shown below the horizontal dashed line in both A and B. Each symbol represents one case.

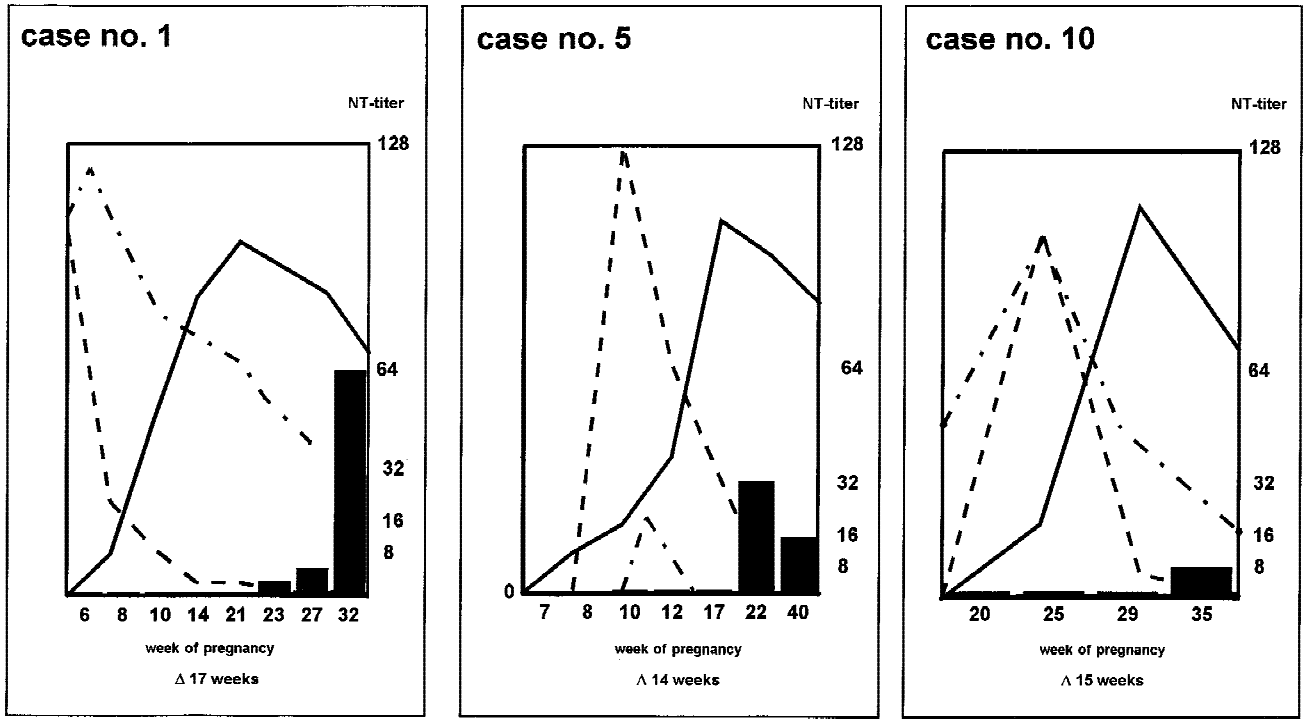

Fig. 2. NT-seroconversion of 3 pregnant women with well-documented seroconversion. The IgM-specific response was obtained with two tests and is shown by dashed lines (CMV-IgM ELA (in-house), — — —; ETI CYTOK-M reverse (Sorin), —·—·—). Solid line represents the IgG response. Bars indicate development of neutralizing antibodies. The time interval between the first HCMV-positive serum sample and the NT-seroconversion was calculated for each case.

positive serum sample was obtained (case nos. 1 and 10), and in case number 5, the seroconversion occurred between gestational weeks 7–8.

In contrast, all of the 235 serum samples from 162 pregnant women of group II, with serologically-confirmed previous infection, had specific neutralizing antibody titers. Figure 1B shows 12 representative cases of this group, in which neutralizing antibodies were detected in all follow-up sera. However, the titers ranged considerably from high to low, and were independent of the strength of the IgG response. There were no apparent differences in titers for sera with long-persisting IgM antibodies or those which were IgM-negative.

The detection of neutralizing antibodies in follow-up sera of group III is presented in Table I. All of these cases were suspected of being acute primary infections because of a positive IgM-response in two IgM-tests or a highly positive IgM-response in at least one of two tests, but definitive conclusions could not be obtained by standard serological methods.

In general, the neutralizing-antibody pattern in group III was equivalent to that of group I (seroconversion) and distinguishable from that of women previously infected (group II). As in group I, the first serum sample was consistently negative and the neutralizing-antibody response was found to increase after some weeks. The appearance of neutralizing antibodies correlated in the majority of cases of group I and III with a decline of the IgM antibodies. In both groups I and III, particularly low neutralizing antibody titers were found.

The outcome of pregnancy was studied to determine the relationship of maternal neutralizing titers with intrauterine transmission of the virus to the fetus. The outcome of pregnancy was known in 34 of the women. The optimal method for diagnosis of congenital infection is detection of HCMV in urine or saliva by isolation of the virus in cell culture or by the rapid shell vial assay, since IgM antibodies may be absent in >35% of congenitally infected infants [Enders, 1998]. Positive results, e.g., in urine specimens obtained from asymptomatic infants after the third week of life, should be interpreted with caution because intrauterine infection cannot be differentiated from infections acquired perinatally after this time period [Sawyer et al., 1987]. Urine samples were collected within the first 2 weeks of life from 11 newborns of mothers from group I, from 5 newborns of mothers of group II, and from 13 of the liveborn infants from mothers of group III. Six out of 11 neonates from women with seroconversion (group I) were asymptomatically infected, and 3 of the 5 fetuses from whom abortion materials were available for virologic studies as well as 3 of the 15 liveborn infants from mothers with suspected acute primary infection (group III) also proved to be congenitally infected. Three of the aborted fetuses (Table I, cases 5, 20, and 21) with ultrasonic abnormalities and HCMV detection in amniotic fluid and fetal blood had severe manifestations of cytomegalic inclusion disease (CID). In the 5 investi-

gated newborns of mothers previously infected, viruria was not detectable.

In contrast to other investigators [Tanaka et al., 1991; Boppana and Britt, 1995], no relationship was found between outcome of pregnancy and neutralizing titers of transmitting and nontransmitting mothers.

DISCUSSION

A reliable method has been described for differentiating acute primary from recurrent HCMV infection. The neutralizing antibody response was investigated in 370 sera from 198 pregnant women against the HCMV reference strain AD169 without complement enhancement. As described by Spiller et al. [1997], neutralizing titers with and without complement enhancement were compared (data not shown). Complement was ineffective at mediating neutralization of the virus in the absence of specific CMV-neutralizing antibodies, whereas adding human complement to heat-inactivated neutralization test (NT)-positive serum increased the neutralizing activity in a few cases. Repeated testing for neutralizing capacity of follow-up sera after acute primary infection revealed that the range of neutralizing titers varied between ± one dilution step, whereas negative sera always remained negative. In order to address the strain specificity of neutralizing antibodies, a microneutralizing assay was carried out with the Towne strain (data not shown). In accordance with Weber et al. [1993], no difference in neutralizing response was observed for the AD169 and Towne strains. Generally, the important practical advantages of the microneutralization assay are its low cost and clear-cut results. However, this technique needs a specialized cell-culture laboratory.

The neutralizing antibody response of pregnant women with well-documented HCMV seroconversion (group I), women infected previously (group II), and pregnant women whose status of infection could not be characterized readily by standard serological methods (group III) were compared. It was found that all HCMV-infected women acquired a neutralizing capacity against AD169, yet the most important observation was that the neutralizing antibody response was absent initially for approximately 15 weeks following infection. This finding supports the suggestion of a delayed synthesis of glycoprotein-specific antibodies which mainly recognize neutralizing epitopes [Schoppel et al., 1997]. In their recent study with recombinant antigens representing the neutralizing epitope sequence of HCMV glycoproteins B (gB, gpUL55) and H (gH, gpUL75), Schoppel et al. [1997] found that the detection of glycoprotein-specific antibodies characterized a previous HCMV-infection, whereas in case of a primary infection, the glycoprotein-specific immune response was absent and first became detectable 50–100 days postinfection. Between 27–100% of the total HCMV neutralizing activity in human sera is directed against gB [Britt et al., 1990] whereas 0–58% of neutralizing antibodies recognize the conformational epitope of gH [Urban et al., 1996]. However, not all sera are able to recognize recombinant antigens, even if they possess high titers of antibodies against whole virus [Kropff et al., 1993]. The detection of neutralizing antibodies by the microneutralization assay is therefore a sensitive alternative, particularly since commercial kits for the detection of glycoprotein-specific antibodies with recombinant antigens are not currently available.

The next issue was to determine whether the estimation of neutralizing antibodies in sera of women whose first samples were suspect serologically (group III) would be helpful for diagnosis of a primary infection. High positive IgM titers in one or both IgM-tests were found in 79% of the cases. This finding, together with a decline of IgM-titer in follow-up sera, is strongly suggestive of acute primary HCMV-infection. All of these patients had an NT-seroconversion confirming the suspicion. Cases 6, 12, 17, 19, and 20 (see Table I) were more difficult to differentiate by standard serological methods because of their low positive IgM-titers in the first serum sample. Here, the discrimination between primary and past infection was only possible using the microneutralization assay.

The results of the study show that the examination of maternal neutralizing antibodies by the simple microneutralization assay is most useful when basic serological tests suggest primary infection during pregnancy. In such cases, a negative NT-titer proved to be a reliable indication for acute primary infection. Diagnosis of acute primary infection, particularly during the first and second trimesters, implicates further measures (e.g., ultrasonography, amniocentesis, and fetal blood sampling). On the other hand, a positive NT-titer in conjunction with other serological data can exclude a recent primary infection in the last 15 weeks and helps to prevent unnecessary invasive prenatal diagnosis [Enders, 1998].

In contrast to other studies, a correlation was not observed between maternal neutralizing antibody titers and intrauterine transmission of the virus to the fetus [Tanaka et al., 1991; Boppana and Britt, 1995]. As indicated by the limited numbers of newborns studied, the NT-titers of mothers who delivered uninfected offspring corresponded to those of transmitters. To substantiate this finding, additional data of mothers with acute primary infection in pregnancy and the conditions of their newborns are needed. In cases of documented seroconversion (group I), we observed HCMV transmission to the fetuses/newborns in 6 out of 11 pregnancies (54%), and in mothers of group III intrauterine transmission was documented in 6 out of 18 pregnancies (3 aborted fetuses and 3 out of 15 liveborn infants) (33%), but transmission was not observed in the 5 newborns from previously infected mothers of group II. The overall transmission rate for groups I and III is 43%, which is consistent with the findings of several investigators of a mean rate of 40% [Stagno et al., 1986; Demmler, 1991].

The microneutralization assay is useful for differentiating primary from recurrent or previous infection in pregnant women. It may also serve as a reference test

TABLE I. Suspected Acute Primary HCMV Infection in Pregnant Women (Group III)*

Case no.	WOP	Enzygnost anti-IgG EIA, Behring (units/l)[a]	CMV-IgM ELA (in-house) (titer)[a]	ETI CYTOK-M reverse, Sorin (index)[b]	Neutralizing antibody titer[c]	Outcome of pregnancy for fetus/newborn
1	10	4,698	1:4,096	1.808	Neg	
	19	7,562	1:2,048	0.456	Neg	
	24	5,294	1:1,024	Neg	1:8	
	28	5,143	1:512	Neg	1:4	
	a.d.	9,074	1:128	Neg	1:16	Not infected
2	9	2,421	1:1,024	1.651	Neg	
	11	2,119	1:1,024	1.477	Neg	
	14	3,728	1:4,096	1.017	1:16	
	17	1,816	1:1,024	0.702	1:16	
	21	4,236	1:256	Neg	1:32	
	23	2,950	1:64	n.d.	1:512	Pregnancy in progress
3	7	4,992	1:8,192	2.85	Neg	
	9	8,318	1:1,024	2.09	Neg	
	14	9,074	1:256	0.529	Neg	
	a.d.	7,411	1:64	Neg	1:32	Not infected
4	11	1,892	1:16,000	2.500	Neg	
	14	2,648	1:16,000	1.187	Neg	
	a.d.	6,201	<1:32	Neg	1:32	Not infected
5	29	4,816	1:4,096	1.625	Neg	Microcephalus, therapeutic abortion/CI, CID
6	19	2,270	1:128	0.464	Neg	
	a.d.	1,287	<1:32	Neg	1:32	Not infected
7	11	4,236	1:8,192	1.609	Neg	
	13	4,992	1:2,048	0.775	1:8	
	18	8,318	1:256	0.445	1:16	CI
8	20	1,212	1:8,192	3.096	Neg	
	22	2,346	1:2,048	2.099	Neg	
	29	5,672	1:2,048	Neg	Neg	
	a.d.	2,950	<1:32	Neg	1:16	Not infected
9	7	n.d.	1:1,024	1.195	Neg	
	9	n.d.	1:1,024	0.813	Neg	
	11	n.d.	1:256	0.918	1:4	
	17	5,067	1:64	0.513	1:8	Pregnancy in progress
10	10	5,143	1:4,096	Neg	Neg	
	16	5,294	1:4,096	0.316	Neg	
	23	4,387	1:2,048	Neg	1:32	CI
11	7	433	1:32,000	3.456	Neg	
	8	667	1:64,000	3.154	Neg	Missed abortion/no material available
12	21	3,404	1:128	0.315	Neg	No data available
13	5	887	1:64,000	Neg	Neg	
	8	3,253	1:8,192	2.780	Neg	
	13	4,992	1:2,048	1.305	1:8	
	14	7,033	1:256	0.855	1:16	
	22	5,370	<1:32	Neg	1:64	
	32	6,050	<1:32	Neg	1:512	Not infected
14	14	6,428	1:128	1.366	Neg	
	16	3,631	<1:32	0.696	Neg	
	20	3,631	<1:32	0.866	1:16	
	a.d.	3,858	<1:32	Neg	1:512	CI
15	19	3,555	1:512	0.800	Neg	
	21	3,933	1:64	0.471	1:8	
	a.d.	7,489	<1:32	Neg	1:32	Not infected
16	31	1,590	1:16,000	1.352	Neg	
	35	2,346	1:8,192	0.625	1:16	No data available
17	15	4,765	1:512	0.786	Neg	
	22	5,370	1:64	Borderline	Neg	
	26	5,445	<1:32	Neg	1:32	
	a.d.	5,596	<1:32	Neg	1:8	Not infected
18	20	1,514	1:2,048	2.534	Neg	
	28	1,590	1:64,000	2.638	Neg	Hydramnion, not infected
	a.d.	2,412	1:8,192	0.499	1:8/16	

(continued)

TABLE I. Continued

Case no.	WOP	Enzygnost anti-IgG EIA, Behring (units/l)[a]	CMV-IgM ELA (in-house) (titer)[a]	ETI CYTOK-M reverse, Sorin (index)[b]	Neutralizing antibody titer[c]	Outcome of pregnancy for fetus/newborn
19	21	4,311	1:2,048	Neg	Neg	
	23	5,596	1:64	Neg	1:16	
	27	3,555	1:128	Neg	1:64	
	34	4,009	1:128	Neg	1:512	
	a.d.	2,724	<1:32	Neg	1:512	Not infected
20	15	3,026	1:1,028	0.651	Neg	
	18	1,816	1:512	0.398	Neg	
	20	3,404	1:128	Borderline	1:8	
	22	3,661	1:256	Borderline	1:4	
	23	9,318	1:256	Borderline	1:128	CI/IUD/CID
21	10	1,590	1:4,096	0.468	Neg	
	12	2,194	1:1,024	Neg	1:8	
	16	2,346	1:256	Neg	1:64	
	24	9,830	1:128	Neg	1:512	Therapeutic abortion/CI, CID
22	20	2,194	1:16,000	1.178	Neg	
	a.d.	3,631	1:256	Borderline	1:32	Not infected
23	8	720	1:32,000	3.459	Neg	
	12	4,916	1:16,000	3.555	Neg	Missed abortion/no material available
24	12	5,294	1:1,024	1.322	Neg	
	14	8,318	1:512	0.884	Neg	
	19	9,074	1:128	Neg	1:4	Not infected

*Neg, negative; n.d., not determined; a.d., at delivery (usually week 36–42 of gestation); WOP; week of pregnancy; IUD, intrauterine death; CI, congenitally infected; CID, cytomegalic inclusion disease.
[a]HCMV IgG titers of ⩽180 and IgM-ELA titers of ⩽1:32 were considered to be negative.
[b]ETI CYTOK-M reverse, the cutoff index was defined as mean OD cutoff control ±10%.
[c]Virus neutralization was calculated as a 50% reduction in number of infected cells 24 hr after infection, as compared to a control with negative serum added. Results are given as an average of duplicate wells, and titers of <1:4 were considered to be negative.

for evaluating newly developed diagnostic assays such as immunoblots or ELISA-based on recombinant antigens. Furthermore, the microneutralization assay could play a role in the detection of the neutralizing-antibody response induced by vaccination with live or subunit HCMV vaccines. The presence of neutralizing antibodies will be one of the important hallmarks for possible long-term protection against HCMV infection following vaccination [Adler et al., 1995].

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